CHEEVER et al.   PATENT
Application No.: 09/493,480
Page 4

REMARKS

I.   **Status of the Claims**

Claims 1-88 were originally filed and later canceled. Claims 89-124 were subsequently added. Upon entry of the present amendment, claims 93-130 are under examination.

Claims 93 and 103 are drawn to nucleic acids encoding fusion proteins comprising a HER-2/neu extracellular domain (ECD) and a HER-2/neu phosphorylation domain (PD and ΔPD, respectively). Prior to the present amendment, the claims recite hybridization under stringent conditions to the complement of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:6 (human ECD-PD fusion protein) and SEQ ID NO:7 (human ECD-ΔPD fusion protein), respectively. As amended, claims 93 and 103 in addition recite separate hybridization to the complement of a nucleic acid encoding the amino acid sequences of SEQ ID NO:3 (human ECD) and SEQ ID NO:4 (human PD), and separate hybridization to the complement of a nucleic acid encoding the amino acid sequences of SEQ ID NO:3 (human ECD) and SEQ ID NO:5 (human ΔPD), respectively. The support for these reference amino acid sequences can be found in the specification, e.g., on page 13 line 20 and lines 27-28. The newly added claim 125 recites "lipid," supported by the specification, e.g., on page 50 lines 29-32. New claim 126 recites "adenoviral vector," which finds support in the specification, e.g., on page 49 lines 9-11. New claims 127-132 recite physiologically acceptable carriers or diluents and/or immunostimulatory substances, also supported by the specification, e.g., on page 51 line 19 to page 51 line 3. No new matter is introduced.

The amendment to the specification merely deletes the reference to certain adjuvants and adds no new matter.

II.   **Claim Rejections**

Claims 93, 97-1-3, 107-116, 121, and 124 were rejected under 35 USC §112 first paragraph for alleged failure to meet the written description requirement. Applicants respectfully traverse the rejections in light of the present amendment.

CHEEVER et al.   <ins>PATENT</ins>
Application No.: 09/493,480
Page 5

        Prior to the instant amendment, claims 93 and 103 are drawn to nucleic acids capable of hybridizing under stringent conditions to the complement of a nucleic acid encoding the amino acid sequence of SEQ ID NO:6 and SEQ ID NO:7 (i.e., human ECD-PD fusion protein and human ECD-ΔPD fusion protein), respectively. The Examiner asserted that the claims as drafted encompass a large genus of nucleic acids of any size and with homology to only a small number of nucleotide residues within a reference sequence, and that the specification lacks information to convey to one of ordinary skill in the art that the applicant had possession of the claimed genus of nucleic acids at the time the instant application was filed.

        Applicants understand that the Examiner is concerned with small fragments of polynucleotides that are within the scope of the claims yet do not have a sufficient overall sequence homology to the reference sequences, and respectfully note that because the claims recite "a fusion protein comprising a HER-2/neu extracellular domain fused to a HER-2/neu phosphorylation domain" in addition to the functional limitation of "capable of producing an immune response in a warm-blooded animal," most, if not all, inoperable small fragments of polynucleotides should be excluded from the claim scope.

        To expedite prosecution, however, Applicants have amended claims 93 and 103 to recite additional limitations that the claimed nucleic acids are capable of hybridizing, under stringent conditions, separately to two additional reference sequences: the complements of an ECD-encoding polynucleotide (SEQ ID NO:3) and a PD- (or ΔPD-) encoding polynucleotide (SEQ ID NO:4 or SEQ ID NO:5). This recitation requires the claimed nucleic acids to encode fusion proteins with sufficient sequence homology in both the ECD region and the PD/ΔPD region. The claims as amended thus require an overall sequence homology in the polypeptides encoded by the claimed nucleic acids, as well as sequence homology within each of the extracellular domain and the phosphorylation domain. This claim language ensures the polypeptides encoded by the claimed nucleic acids to be the fusion proteins of ECD and PD or ECD and ΔPD with

CHEEVER et al.
Application No.: 09/493,480
Page 6

PATENT

two appropriate domains. Small polynucleotides homologous to only a small region within either one of the ECD region and the PD/ΔPD region are therefore excluded from the amended claims.

The pending claims as amended fully comply with the requirements for written description of a chemical genus as set forth in *University of California v. Eli Lilly & Co.*, 43 USPQ2d 1398 (Fed. Cir. 1997). As described by the Federal Circuit in *Lilly*, "[a] description of a genus of cDNAs may be achieved by means of . . . a recitation of structural features common to the members of the genus . . . ." *Lilly*, 43 USPQ2d at 1406. Furthermore, the court in *Fiers v. Revel* stated that an adequate written description "requires a precise definition, such as by structure, formula, chemical name, or physical properties." *Fiers*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993). Finally, the MPEP states that structural formulas provide a convenient method of demonstrating possession of specific molecules. MPEP §2163.

On the other hand, proper description of functional features of a claimed invention can play an important role in satisfying the written description requirement. The Federal Circuit recently stated that "*Eli Lilly* did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." *Amgen Inc. v. Hoechst Marion Roussel Inc.*, 65 USPQ2d 1385, 1398 (Fed. Cir. 2003).

With regard to the claimed nucleic acids, claims 93 and 103 (and hence their dependent claims) set forth both functional elements, *e.g.*, encoding a fusion protein that is capable of producing an immune response in a warm-blooded animal, as well as structural elements, *e.g.*, capable of hybridizing to a reference sequence under specified hybridization conditions. Applicants submit, therefore, that the claimed nucleic acids are thereby defined via shared functional and structural properties.

The ability for a nucleic acid to hybridize under given conditions to a reference polynucleotide sequence, such as a DNA sequence encoding a particular amino

CHEEVER et al.   PATENT
Application No.: 09/493,480
Page 7

acid sequence, is a physical/structural property of the nucleic acid, because it relies upon

the nucleotide sequence of the molecule. *See, e.g.,* Sambrook et al., *Molecular Cloning:*

A Laboratory Manual, pages 9.47-9.51 (2nd ed. 1989), attached as Exhibit A; Stryer,

Biochemistry, pages 80-82 (3rd ed. 1988), attached as Exhibit B. As described in Stryer,

the transition between hybridization and melting of complementary nucleic acid strands

is abrupt and largely sequence dependent. When the temperature of hybridization is

provided, one of skill in the art would be able to predict whether or not a given sequence

would hybridize to a reference sequence (*see, e.g.,* equations provided in Sambrook,

supra).

The functional features of the claimed nucleic acids are also provided:

each encodes a fusion protein capable of producing an immune response in a warm-

blooded animal, which can be readily tested by one of ordinary skill in the art using well

established, routinely practiced techniques.

Thus, both structural and functional features commonly shared by the

claimed genus have been described in detail, which "clearly allow persons of ordinary

skill in the art to recognize that [the applicant] invented what is claimed." *Vas-Cath Inc.*

v. Mahurkar, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). Such description is consistent

with the standards set forth in both *Lilly* and *Amgen.* As such, Applicants submit the

written description requirement is met and respectfully request the withdrawal of the

rejections under 35 USC §112 first paragraph.

III.   Objections

Claims 94-96, 104-106, 117-120, 122, and 123 have been objected to for

being dependent from rejected claims 93 and 103. Applicants believe that the present

amendment has removed the basis for the claim rejections and thus rendered the
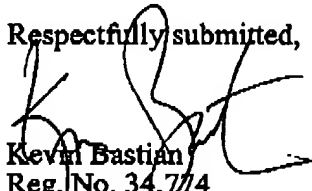
objections moot.

CHEEVER et al.   <u>PATENT</u>
Application No.: 09/493,480
Page 8

<div align="center"><u>CONCLUSION</u></div>

In view of the foregoing, Applicants believe all claims now pending in this
Application are in condition for allowance and an action to that end is urged. If the
Examiner believes a telephone conference would aid in the prosecution of this case in any
way, please call the undersigned at 415-576-0200.

Respectfully submitted,

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CHEEVER et al.
Application No.: 09/493,480
Page 9

APPENDIX A

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

In the specification on page 52, lines 4-10:

--Other preferred adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), [the SBAS series of adjuvants (*e.g.,* SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium),] Detox (Corixa Corporation, Hamilton, MT), RC-529 (Corixa, USA) and Aminoalkyl glucosaminide 4-phosphates (AGPs).

In a preferred embodiment, the adjuvant is [SBAS-2 (*See, e.g.,*] described in EP 735898B1[)].--

IN THE CLAIMS:

93. (twice amended) An isolated nucleic acid encoding a fusion protein comprising a HER-2/neu extracellular domain fused to a HER-2/neu phosphorylation domain, wherein the nucleic acid hybridizes under stringent conditions to the complement of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 6, and separately to the complement of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:3, and separately to the complement of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO:4, wherein the hybridization reaction is incubated in a solution comprising 5x SSC at a temperature of 50-65°C and washed in a solution comprising 0.2x SSC and 0.1% SDS at a temperature of 65°C, and wherein the protein is capable of producing an immune response in a warm-blooded animal.

CHEEVER et al.   PATENT
Application No.: 09/493,480
Page 10

          103.   (twice amended) An isolated nucleic acid encoding a fusion

protein comprising a HER-2/neu extracellular domain fused to a fragment of the HER-

2/neu phosphorylation domain, wherein the nucleic acid hybridizes under stringent

conditions to the complement of a nucleic acid encoding the amino acid sequence of SEQ

ID NO:7, and separately to the complement of a nucleic acid sequence encoding the

amino acid sequence of SEQ ID NO:3, and separately to the complement of a nucleic

acid sequence encoding the amino acid sequence of SEQ ID NO:5, wherein the

hybridization reaction is incubated in a solution comprising 5x SSC at a temperature of

50-65°C and washed in a solution comprising 0.2x SSC and 0.1% SDS at a temperature

of 65°C, and wherein the protein is capable of producing an immune response in a warm-

blooded animal.

EXHIBIT A

2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

J. Sambrook
UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch
GENETICS INSTITUTE

T. Maniatis
HARVARD UNIVERSITY

YBRIDIZATION OF RADIOLABELED PROBES
IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)

- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)

- Degree and method of agitation (continuous shaking or stationary)

- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix

- Concentration of the labeled probe and its specific activity

- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or poly-ethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids

- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.

2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.

3. Continual movement of the probe solution across the filter is un-necessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneous-ly, agitation is advisable to prevent the filters from adhering to one another.

4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

Analysis and Cloning of Eukaryotic Genomic DNA **9.47**

immobilized nucleic acid and its availability for hybridization are un-known. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1-3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by pre-hybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

9.48 *Analysis and Cloning of Eukaryotic Genomic DNA*

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TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented, salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia) 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Pentex Fraction V), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% non-fat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into hybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The solution is adjusted to 0.1 M NaCl and extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered and the DNA is sheared by passing it 1 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation an redissolved at a concentration of 10 mg/ml in water. The OD^{260} of the solution i determined and the exact concentration of the DNA is calculated. The solution then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use the solution is heated for 5 minutes in a boiling-water bath and then chilled quickl in ice water. Denatured, fragmented salmon sperm DNA should be used at concentration of 100 μg/ml in hybridization solutions.

Analysis and Cloning of Eukaryotic Genomic DNA **9.4**

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962)

9.50   *Analysis and Cloning of Eukaryotic Genomic DNA*

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\% G + C) - 0.63(\% \text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.
This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.

- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.
Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\%G + C) + 11.8(\%G + C)^2 - 0.35(\% \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\%G + C) + 11.8(\%G + C)^2 - 0.50(\% \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).
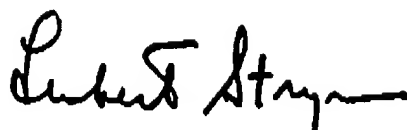
The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

EXHIBIT B

BIOCHEMISTRY

THIRD EDITION

LUBERT STRYER

STANFORD UNIVERSITY

W. H. FREEMAN AND COMPANY / NEW YORK

80

Part I
MOLECULAR DESIGN OF LIFE

The distribution of ^{14}N and ^{15}N was revealed by the newly developec technique of *density-gradient equilibrium sedimentation*. A small amount o. DNA was dissolved in a concentrated solution of cesium chloride hav ing a density close to that of the DNA (~ 1.7 g cm^{-3}). This solution wa centrifuged until it was nearly at equilibrium. The opposing processe of sedimentation and diffusion created a gradient in the concentratioi of cesium chloride across the centrifuge cell. The result was a stabl density gradient, ranging from 1.66 to 1.76 g cm^{-3}. The DNA mole cules in this density gradient were driven by centrifugal force into th region where the solution's density was equal to their own. High molecular-weight DNA yielded a narrow band that was detected by it absorption of ultraviolet light. A mixture of ^{14}N DNA and ^{15}N DN/ molecules gave clearly separate bands because they differ in density b about 1% (Figure 4-13).
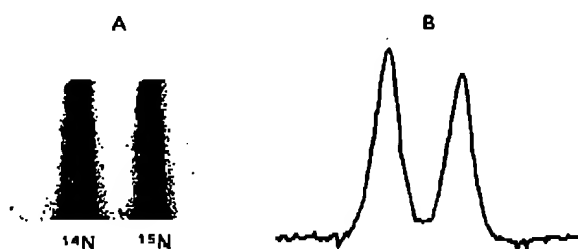

Figure 4-13
Resolution of ^{14}N DNA and ^{15}N DNA by density-gradi-ent centrifugation: (A) ultraviolet absorption photograph of a centrifuge cell; (B) densitometric tracing of the ab-sorption photograph. [From M. Meselson and F. W. Stahl. *Proc. Nat. Acad. Sci.* 44(1958):671.]

DNA was extracted from the bacteria at various times after they wei transferred from a ^{15}N to a ^{14}N medium. Analysis of these samples l the density-gradient technique showed that there was a single band DNA after one generation (Figure 4-14). The density of this band w precisely halfway between those of ^{14}N DNA and ^{15}N DNA. *The absen of ^{15}N DNA indicated that parental DNA was not preserved as an intact unit . replication.* The absence of ^{14}N DNA indicated that all of the daught DNA molecules derived some of their atoms from the parent DN This proportion had to be one-half, because the density of the hybr DNA band was halfway between those of ^{14}N DNA and ^{15}N DN

After two generations, there were equal amounts of two bands DNA. One was hybrid DNA, the other was ^{14}N DNA. Meselson ai Stahl concluded from these incisive experiments *"that the nitrogen o, DNA molecule is divided equally between two physically continuous subun: that, following duplication, each daughter molecule receives one of these; o that the subunits are conserved through many duplications."* Their resu agreed perfectly with the Watson-Crick model for DNA replicati (Figure 4-15).

THE DOUBLE HELIX CAN BE REVERSIBLY MELTED

The two strands of a DNA helix readily come apart when the hydrof bonds between its paired bases are disrupted. This can be accomplist

Generations

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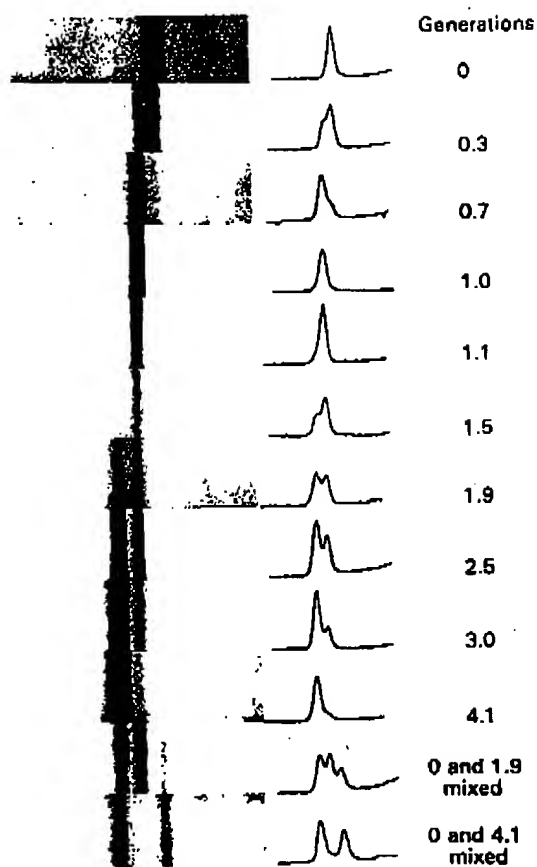
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4.1

0 and 1.9
mixed

0 and 4.1
mixed

Figure 4-14
Detection of semiconservative replication in *E. coli* by density-gradient centrifugation. The position of a band of DNA depends on its content of ^{14}N and ^{15}N. After 1.0 generation, all of the DNA molecules are hybrids containing equal amounts of ^{14}N and ^{15}N. No parental DNA (^{15}N) is left after 1.0 generation. [From M. Meselson and F. W. Stahl. *Proc. Nat. Acad. Sci.* 44(1958):671.]

Original parent molecule

First generation daughter molecules

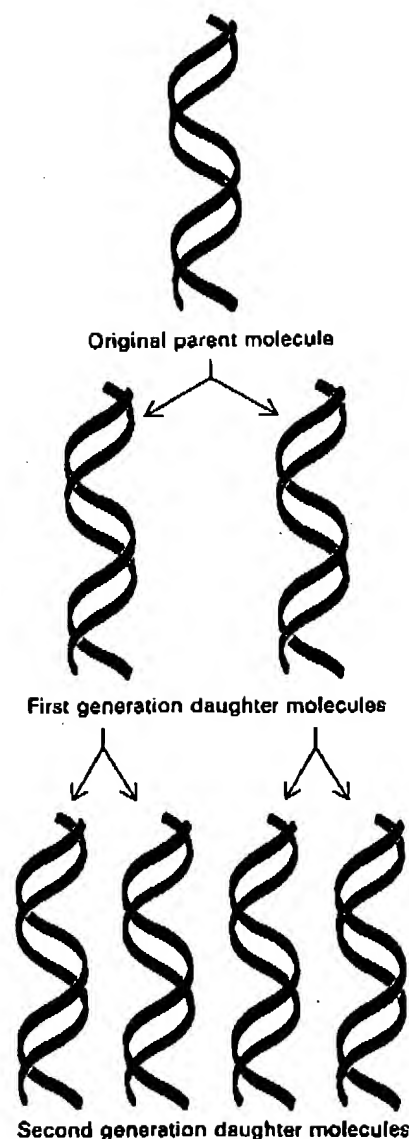Second generation daughter molecules

Figure 4-15
Schematic diagram of semiconservative replication. Parental DNA is shown in green and newly synthesized DNA in red. [After M. Meselson and F. W. Stahl. *Proc. Nat. Acad. Sci.* 44(1958):671.]
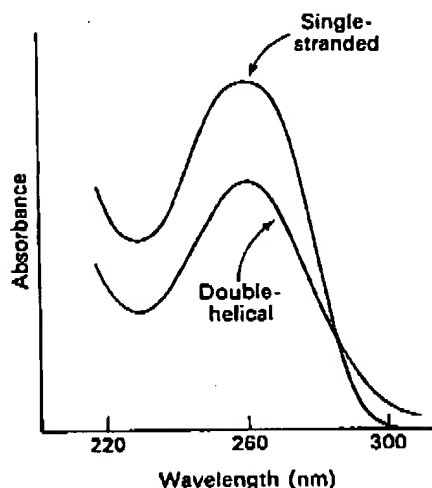
82

Part I
MOLECULAR DESIGN OF LIFE

Figure 4-16
The absorbance of a DNA solution at wavelength 260 nm increases when the double helix is melted into single strands.
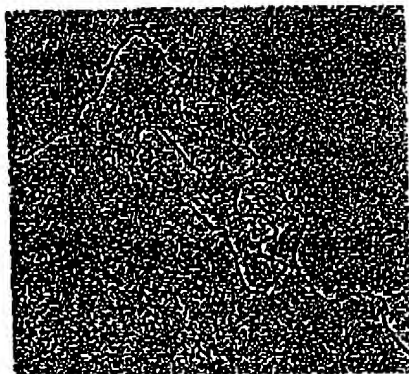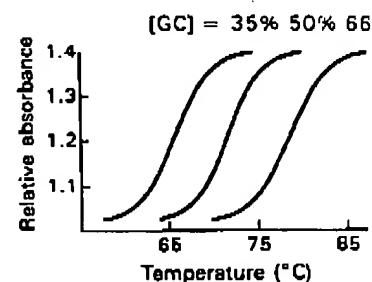

Figure 4-18
Electron micrograph of a DNA molecule partly unwound by alkali. Single-stranded regions appear as loops that stain less intensely than double-stranded segments. These unwound regions are rich in AT base pairs. One of them is marked by an arrow. [From R. B. Inman and M. Schnos. *J. Mol. Biol.* 49(1970):93.]

by heating a solution of DNA or by adding acid or alkali to ionize bases. The unwinding of the double helix is called *melting* because occurs abruptly at a certain temperature. The *melting temperature* (T_m) defined as the temperature at which half of the helical structure is lo The abruptness of the transition indicates that the DNA double helix a *highly cooperative structure*, held together by many reinforcing bonds is stabilized by the stacking of bases as well as by base pairing. T melting of DNA is readily monitored by measuring its absorbance light at wavelength 260 nm. The unstacking of the base pairs results increased absorbance, an effect called *hyperchromism* (Figure 4-16).

The melting temperature of a DNA molecule depends markedly its base composition. DNA molecules rich in GC base pairs have higher T_m than those having an abundance of AT base pairs (Figt 4-17). In fact, the T_m of DNA from many species varies linearly with (

Figure 4-17
DNA melting curves. The absorbance relative to that at 25°C is plotted against temperature. (The wavelength of the incident light was 260 nm.) The T_m is 69°C for *E. coli* DNA (50% GC pairs) and 76°C for *P. aeruginosa* DNA (68% GC pairs).

content, rising from 77°C to 100°C as the fraction of GC pairs incre: from 20% to 78%. GC base pairs are more stable than AT pairs beca their bases are held together by three hydrogen bonds rather thar two. In addition, adjacent GC base pairs interact more strongly \ one another than do adjacent AT base pairs. Hence, *the AT-rich reg of DNA are the first to melt* (Figure 4-18). The double helix is melte vivo by the action of specific proteins (p. 672).

Separated complementary strands of DNA spontaneously reasso to form a double helix when the temperature is lowered below T_m. renaturation process is sometimes called *annealing*. The facility which double helices can be melted and then reassociated is crucia the biological functions of DNA.

DNA MOLECULES ARE VERY LONG

A striking characteristic of naturally occurring DNA molecules is length. DNA molecules must be very long to encode the large nu of proteins present in even the simplest cells. The *E. coli* chromos for example, is a single molecule of double-helical DNA consisti four million base pairs. The mass of this DNA molecule is $2.6 \times 1($ It has a *highly asymmetric shape* when taken out of the cell. The leng *E. coli* DNA is 14×10^6 Å, but its diameter is only 20 Å. The 1. length of this DNA molecule corresponds to a macroscopic dimer whereas its width of 20 Å is on the atomic scale. Bruno Zimm f that the largest chromosome of *Drosophila melanogaster* contains a : DNA molecule of 6.2×10^7 base pairs, which has a length of 2.